The nonhomologous DNA end joining pathway is important for chromosome stability in primary fibroblasts

Zarir E. Karanjawala*†, Ulf Grawunder†‡, Chih-Lin Hsieh§¶ and Michael R. Lieber*¶¥

There are two types of chromosome instability, structural and numerical, and these are important in cancer. Many structural abnormalities are likely to involve double-strand DNA (dsDNA) breaks. Nonhomologous DNA end joining (NHEJ) and homologous recombination are the major pathways for repairing dsDNA breaks. NHEJ is the primary pathway for repairing dsDNA breaks throughout the G0, G1 and early S phases of the cell cycle [1]. Ku86 and DNA ligase IV are two major proteins in the NHEJ pathway. We examined primary dermal fibroblasts from mice (wild type, Ku86$^{+/-}$, Ku86$^{-/-}$, and DNA ligase IV$^{+/-}$) for chromosome breaks. Fibroblasts from Ku86$^{+/-}$ or DNA ligase IV$^{+/-}$ mice have elevated frequencies of chromosome breaks compared with those from wild-type mice. Fibroblasts from Ku86$^{-/-}$ mice have even higher levels of chromosome breaks. Primary pre-B cells from the same animals did not show significant accumulation of chromosome breaks. Rather the pre-B cells showed increased cell death. These studies demonstrate that chromosome breaks arise frequently and that NHEJ is required to repair this constant spontaneous damage.

Addresses: *Department of Pathology, §Department of Urology, ¶Department of Biochemistry and Molecular Biology and ¥Department of Molecular Microbiology and Immunology, Norris Comprehensive Cancer Center, Rooms 5420 and 5428, University of Southern California School of Medicine, 1441 Eastlake Avenue, MS 73, Los Angeles, California 90033, USA.

Present address: ‡Basel Institute of Immunology, Grenzachestrasse 487, CH-4005 Basel, Switzerland.

†Z.E.K. and U.G. contributed equally to this work.

Correspondence: Michael R. Lieber or Chih-Lin Hsieh
E-mail: lieber@hsc.usc.edu, hsieh_c@froggy.hsc.usc.edu

Received: 30 September 1999
Revised: 1 November 1999
Accepted: 15 November 1999

Published: 6 December 1999

Current Biology 1999, 9:1501–1504

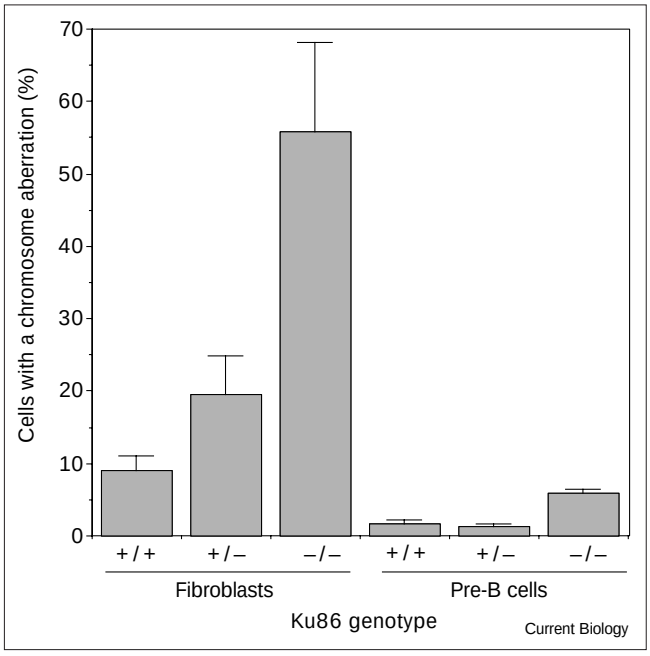
0960-9822/99/$ – see front matter

Results and discussion

Fibroblasts from Ku86$^{+/-}$, Ku86$^{-/-}$ and Lig4$^{+/-}$ mice have marked increases in chromosome breaks

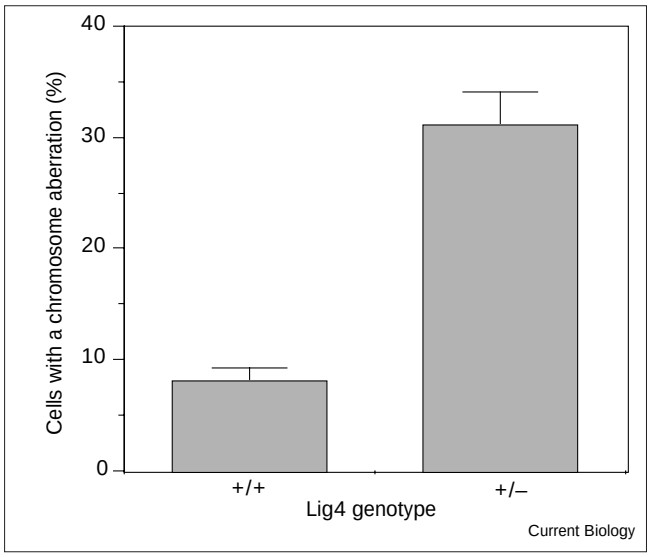
To determine whether a defective NHEJ pathway would affect chromosome stability, we isolated primary dermal fibroblasts from 4 month old wild-type, Ku86$^{+/-}$, Ku86$^{-/-}$ and DNA ligase IV$^{+/-}$ (Lig4$^{+/-}$) mice [2,3], and examined metaphase spreads from these cells after nine days in culture. Fibroblasts from wild-type mice had chromosome breaks in 9 ± 2% of the cells (Figure 1), which is slightly higher than the level observed in a previous study in which murine fibroblasts from young mice had breaks in approximately 5% of their cells [4]. Strikingly, in the NHEJ mutant cells (Ku86$^{-/-}$), the percentage of fibroblasts with breaks increased to 56 ± 12%. Hence, the absence of Ku86 protein results in a marked increase in the number of spontaneous chromosome breaks in fibroblasts. The percentage

Figure 1



Chromosome breaks are increased in primary fibroblasts that are deficient for Ku86 but in pre-B cells the increase is not as marked. Dermal fibroblasts were grown from tail biopsies from 4-month-old wild-type, Ku86$^{+/-}$, and Ku86$^{-/-}$ mice. After 1–2 passages (corresponding to 10–12 days in culture and roughly 12–15 cell divisions), the cells were treated with colcemid, fixed, and metaphase spreads were prepared. Pre-B cells were grown from 4-month-old wild-type, Ku86$^{+/-}$, or Ku86$^{-/-}$ mice as described in the methods (see Supplementary material), and analyzed at 10–14 days by treatment with colcemid, fixation, and preparation of metaphase spreads. The chromosomes were Wright stained. Cells with at least one chromosome aberration were scored as positive. The ordinate indicates the percentage of cells with at least one chromosome break. Error bars represent the standard deviation.

Figure 2



Chromosome breaks are increased in primary fibroblasts heterozygous for DNA ligase IV. Dermal fibroblasts were grown from tail biopsies from 4-month-old wild-type and Lig4$^{+/-}$ mice. After 1–2 passages (corresponding 10–12 days in culture and roughly 12–15 cell divisions), the cells were treated with colcemid, fixed, and metaphase spreads were prepared. The chromosomes were Wright stained. Cells with at least one chromosome aberration were scored as positive. The ordinate indicates the percentage of cells with at least one chromosome break. Error bars represent the standard deviation.

of heterozygous cells with breaks was intermediate (20 ± 5%) between wild-type and null cells, suggesting that the level of Ku86 in heterozygous cells is only partially effective in repairing spontaneous chromosome breaks. We also observed an increase in chromosome instability in Lig4$^{+/-}$ fibroblasts (Figure 2), suggesting that the chromosome instability observed in the Ku86-deficient cells was not caused by a NHEJ-independent role specific for Ku86. Corresponding data from adult Lig4$^{-/-}$ cells cannot be obtained because these animals are inviable [3].

The structure of the chromosome breaks in wild-type, Ku86$^{+/-}$ and Ku86$^{-/-}$ fibroblasts was similar, even though the quantitative differences were substantial. Nearly 90% of the chromosome structural abnormalities are chromosome or chromatid breaks and gaps (Table 1). The remainder of the events probably arise from dsDNA breaks as well.

Proliferation of the heterozygous Ku86$^{+/-}$ and Lig4$^{+/-}$ fibroblasts are normal

We were interested in determining how the observed chromosomal instability might affect the growth rate of fibroblasts. Previous studies in mouse embryonic fibroblasts have demonstrated impaired growth for Ku70-, Ku86-, and Lig4-deficient cells compared with wild-type cells [3,5,6]. Our data are in agreement with these previous studies. The proliferation of fibroblasts from Ku86 or Lig4 heterozygotes was comparable with wild type, whereas the growth of Ku86$^{-/-}$ fibroblasts was markedly slowed (see Supplementary material). Given that the heterozygous cells with normal growth rates demonstrate an increase in chromosome breaks, it is not likely that the Ku86$^{-/-}$ cells simply accumulate breaks because their growth is slowed.

Frequencies of cell death are not elevated in fibroblasts even among those with slowed growth

We wondered whether the marked chromosomal breakage in NHEJ-deficient fibroblasts would cause them to die. We examined fibroblasts for trypan blue exclusion. The frequency of dead fibroblasts was not increased in the Ku86$^{+/-}$ cells, and was not markedly increased even in the Ku86$^{-/-}$ fibroblasts (Figure 3). The chromosome breaks resulting from Ku86 deficiency therefore do not cause increased cell death in the fibroblasts.

Ku86$^{-/-}$ pre-B cells do not manifest the chromosome breaks observed in Ku86$^{-/-}$ fibroblasts

It was of interest to compare the fibroblasts with precursor lymphocytes from the same animals because lymphocytes must contend with physiological dsDNA breaks, generated

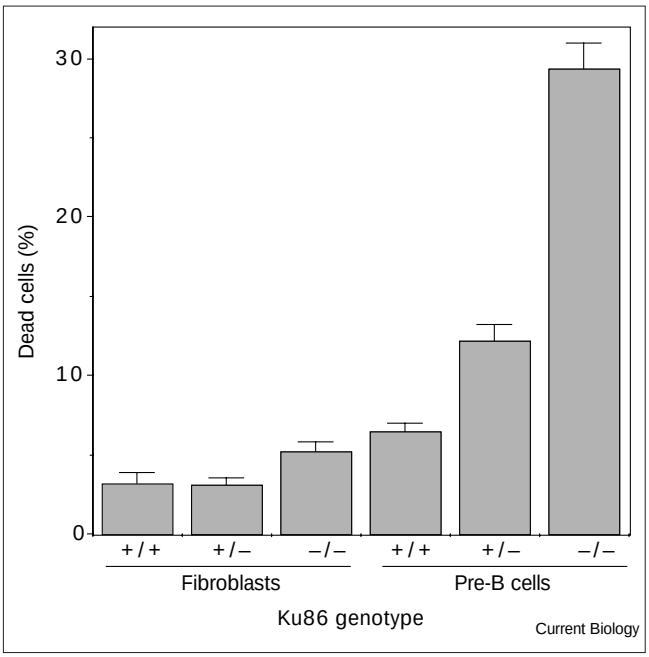
Table 1

Chromosome aberrations in wild-type, Ku86$^{+/-}$, and Ku86$^{-/-}$ murine fibroblasts.

Genotype	CSB/CSG*	CTB/CTG*	Rob*	Chromatid exchange	Other	Total events	Total cells	Average number events/cell†‡	Percentage of cells with events§
	Number of events (percentage of total events)								
Wildtype	1 (6.7)	12 (80.0)	0 (0)	0 (0)	2 (13.3)	15	111	1.5 ± 0.2	9.0 ± 2.3
Ku86$^{+/-}$	20 (19.6)	66 (64.7)	3 (2.9)	1 (1.0)	12 (11.8)	102	392	1.3 ± 0.1	19.6 ± 5.3
Ku86$^{-/-}$	165 (37.0)	240 (53.8)	26 (5.8)	8 (1.8)	7 (1.6)	446	342	2.4 ± 0.3	55.8 ± 12.4

*CSB indicates chromosome break; CSG, chromosome gap; CTB, chromatid break; CTG chromatid gap; and Rob, Robertsonian translocation. †Average number events per cell is the total number of events divided by the number of cells with events. Errors are the standard deviation from multiple experiments. ‡The average number of breaks per cell could be calculated by multiplying CSB/CSG and chromatid exchange events by 2, and Robertsonian translocation events by 4. These events represent either two or four DNA strand breaks, respectively. §The percentage of cells with at least one chromosomal aberration.

Figure 3



Cell death in pre-B lymphocytes deficient for Ku86 is elevated but not for fibroblasts from the same mice. For pre-B cells, the percentage of cell death in cultures was determined by FACS analysis using propidium iodide stained cells (see Supplementary material) and calculating the ratio of FL2 positive/negative events. Fibroblasts were counted in triplicate by trypan blue exclusion at day 9.5, and cell death reported as the percentage of dead cells ± standard deviation.

during V(D)J recombination, in addition to pathological breaks. We cultured and analyzed the metaphase chromosomes of continuously growing interleukin 7 (IL-7)/stromal cell-dependent fetal liver primary pre-B cells from wild-type, Ku86$^{+/-}$, and Ku86$^{-/-}$ mice. As with the corresponding fibroblasts, the lymphocytes with a homozygous deletion of Ku86 grew slowly, although the heterozygous cells were only marginally reduced in growth rate (see Supplementary material). Strikingly, we found that the primary pre-B cells had markedly fewer numbers of chromosome breaks than primary fibroblasts from the same mice for each corresponding genotype (Figure 1).

Ku86$^{+/-}$ and Ku86$^{-/-}$ pre-B cells show markedly elevated cell death relative to wild type

We wondered whether the lack of chromosome breaks in pre-B cells was due to a more rapid triggering to cell death, as has been described for other insults to lymphocytes [7]. Could it be that the chromosome breaks occur, but that the cells in which they occur are rapidly cleared from the culture by cell death? To address this, we determined the frequency of dead cells in optimally growing pre-B cell cultures by fluorescence-activated cell sorter (FACS) analysis (Figure 3). This showed that the frequency of dead cells in Ku86$^{-/-}$ pre-B cell cultures was indeed dramatically increased in Ku86$^{-/-}$ cells. We conclude that the frequency of cell death in pre-B cells is markedly increased in the absence of Ku86.

Interestingly, even pre-B cells from Ku86$^{+/-}$ mice showed a significant elevation in the frequency of dead cells by FACS analysis (Figure 3). The lack of a marked effect on chromosome stability in Ku86-deficient pre-B cells was accompanied by a corresponding increase in cell death as one compares pre-B cells from wild-type, heterozygous, and homozygous mutant Ku86 mice. No significant increase, however, was observed for fibroblasts (Figure 3).

Chromosomal instability and cell-cycle checkpoints

The primary fibroblasts and pre-B cells used in this study were from mice that have intact cell-cycle checkpoints. Hence, it is intriguing that these cells acquire breaks. Relevant to this point, we have crossed Ku86$^{-/-}$ mice with p53 mutant mice to generate mice with both Ku86 and p53 defects. The primary fibroblasts from those mice showed the following chromosomal breakage frequencies at day 9 in culture: 21% of Ku86$^{+/+}$p53$^{-/-}$ fibroblasts had at least one break; 46% of Ku86$^{+/-}$p53$^{-/-}$ fibroblasts had at least one break; and 78% of Ku86$^{-/-}$p53$^{-/-}$ fibroblasts had at least one break. Though the breakage frequencies are worse in the cells from p53$^{-/-}$ mice (regardless of whether they are Ku86$^{+/+}$, Ku86$^{+/-}$, or Ku86$^{-/-}$), these data are striking for the fact that the fraction of cells with breaks is only about 1.4–2-fold higher relative to the corresponding frequencies in Table 1 for Ku86$^{+/+}$, Ku86$^{+/-}$, and Ku86$^{-/-}$ cells. We conclude that chromosome breaks occur, with or without intact checkpoints, at a high frequency when NHEJ is defective.

Biological significance of chromosome instability in the absence of NHEJ

Our results permit several new conclusions. First, primary fibroblasts from Ku86$^{-/-}$ mice have metaphase chromosome breaks in over 55% of the cells, even in the absence of overt ionizing irradiation or oxidative challenge (Figure 1). This indicates that chromosome breaks arise spontaneously and cells require NHEJ to repair them.

Second, the Ku86$^{+/-}$ heterozygous fibroblasts have a chromosome break frequency that is intermediate between the wild-type and Ku86$^{-/-}$ cells. The Lig4$^{+/-}$ cells also have a markedly increased frequency of chromosome breaks relative to that of wild-type cells. This suggests that heterozygous levels of Ku86 and DNA ligase IV in murine fibroblasts are not sufficient to repair many of the spontaneously occurring chromosomal breaks.

Third, pre-B cells show chromosome breaks at frequencies that are 10–20-fold lower than fibroblasts, regardless of their Ku86 genotype. This is most readily explained by the fact that pre-B cells are triggered to die more easily than other cell types [7]. Our analysis of the growth and death

rates are consistent with such an interpretation. Hence, pre-B cells that suffer chromosome breaks might be continuously removed from the pool of proliferating cells relatively quickly, thereby depleting the viable numbers of cells with chromosome breaks that could be visualized.

Chinese hamster ovary (CHO) cell lines that have a genetic mutation in one allele of Ku86 and an unstable CpG methylation suppression of the remaining wild-type allele do not show genetic instability [8,9]. Our study utilizes primary murine cells, not transformed CHO cell lines, which might also contribute to the difference in chromosome stability between our study and earlier ones. Cell lines have often accumulated extensive CpG methylation of their genomes [10], and the resulting chromatin structural change might suppress the accumulation of chromosome breaks. Perhaps more importantly, rapidly dividing transformed cells spend a greater fraction of the cell cycle in late S and G2 phase, in which homologous recombination primarily repairs dsDNA breaks [1].

Our study underscores the potential role of the NHEJ pathway for maintaining chromosome stability in primary fibroblasts. Haploid levels of Ku86 and DNA ligase IV in primary fibroblasts result in increased chromosomal instability. Unlike fibroblasts, lymphoid cells do not manifest chromosomal instability, but rather the lymphoid cells have a very high rate of cell death. We speculate that the increased chromosome instability in primary cells with compromised NHEJ might contribute to the senescence associated with Ku86 mice that was recently documented [11], and might be a potential cause of neuronal loss in Lig4$^{-/-}$ animals [12].

Supplementary material

Supplementary material including additional materials and methods and figures showing growth curves for wild-type, Ku86$^{+/-}$, Ku86$^{-/-}$, and Lig4$^{+/-}$ cells is available at http://current-biology.com/supmat/supmatin.htm.

Acknowledgements

We thank Paul Hasty (Lexicon Genetics, The Woodlands, TX) for the Ku86 mutant mice and Frederick Alt and Karen Frank (Harvard University, Boston, MA) for the Lig4 mutant mice. We thank Robert Tracy for reading the manuscript.Z.E.K. was supported by the National Cancer Institute Program in Molecular Oncology Training Grant (T32 CA09569). This research was supported by National Institutes of Health grants and a Leukemia Society of America Scholar Award (to M.R.L.). M.R.L. is the Rita and Edward Polusky Basic Cancer Research Professor.

References

1. Takata M, Sasaki MS, Sonoda E, Morrison C, Hashimoto M, Utsumi H, *et al.*: Homologous recombination and non-homologous end-joining pathways of DNA double-strand break repair have overlapping roles in the maintenance of chromosomal integrity in vertebrate cells. *EMBO J* 1998, 17:5497-5508.
2. Zhu C, Bogue MA, Lim D-S, Hasty P, Roth DB: Ku86-deficient mice exhibit severe combined immunodeficiency and defective processing of V(D)J recombination intermediates. *Cell* 1996, 86:379-389.
3. Frank KM, Sekiguchi JM, Seidl KJ, Swat W, Rathbun GA, Cheng H-L, *et al.*: Late embryonic lethality and impaired V(D)J recombination in mice lacking DNA ligase IV. *Nature* 1998, 396:173-177.
4. Martin GM, Smith AC, Ketterer DJ, Ogburn CE, Disteche CM: Increased chromosomal aberrations in first metaphases of cells isolated from the kidneys of aged mice. *Isr J Med Sci* 1985, 21:296-301.
5. Gu Y, Seidl K, Rathbun GA, Zhu C, Manis JP, van der Stoep N, *et al.*: Growth retardation and leaky SCID phenotype of Ku70-deficient mice. *Immunity* 1997, 7:653-665.
6. Nussenzweig A, Chen C, da Costa Sores VC, Sanchez M, Sokol K, Nussenzweig MC, *et al.*: Requirement for Ku80 in growth and immunoglobulin V(D)J recombination. *Nature* 1996, 382:551-555.
7. Griffiths SD, Goodhead DT, Marsden SJ, Wright E, Krajewski S, Reed JC, *et al.*: Interleukin-7-dependent B lymphocyte precursor cells are ultrasensitive to apoptosis. *J Exp Med* 1994, 179:1789-1797.
8. Darroudi F, Natarajan AT: Cytological characterization of Chinese hamster ovary X-ray-sensitive mutant cells, xrs5 and xrs6. *Mutat Res* 1987, 177:149-160.
9. Kemp LM, Jeggo PA: Radiation-induced chromosome damage in X-ray sensitive mutants (xrs) of the Chinese hamster ovary cell line. *Mutat Res* 1986, 166:255-263.
10. Antequera F, Boyes J, Bird AP,: High levels of ***de novo*** methylation and altered chromatin structure at CpG islands in cell lines. *Cell* 1990, 62:503-514.
11. Vogel H, Lim D-S, Karsenty G, Finegold M, Hasty P: Deletion of Ku86 causes early onset of senescence in mice. *Proc Natl Acad Sci USA* 1999 96:10770-10775.
12. Gao Y, Sun Y, Frank K, Dikkes P, Fujiwara Y, Seidl K, *et al.*: A critical role for DNA end-joining proteins in both lymphogenesis and neurogenesis. *Cell* 1998, 95:891-902.

Supplementary material

The nonhomologous DNA end joining pathway is important for chromosome stability in primary fibroblasts

Zarir E. Karanjawala, Ulf Grawunder, Chih-Lin Hsieh and Michael R. Lieber

Current Biology 6 December 1999, 9:1501–1504

Materials and methods

Mice

Ku86$^{+/-}$ heterozygous breeding pairs (129SVEV x C57Bl/6) were obtained from Dr. Paul Hasty (Lexicon Genetics, Inc., The Woodlands, TX). Offspring were tested using PCR genotyping (see below) for the presence of the Ku86-deleted allele. Genomic DNA from mouse tails was PCR genotyped for 35–40 cycles with an annealing temperature of 59°C with the primer pairs UG122 (5′-CCA AAG GCC TAC CCG CTT CCA TT) and UG123 (5′-GGT TGC CAG TCA TGC TAC GGT) for the Ku86-targeted allele and UG124 (5′-AGA GGG ACT GCA GCC ATA TTA) and UG125 (5′-GAG AGT CTA CGA CAA CTG TGC) for the wild type allele.

DNA ligase IV$^{+/-}$ (Lig4$^{+/-}$) breeding pairs (129/SV x C57Bl/6) were obtained from Dr. F. Alt (Harvard U., Boston, MA). Offspring were genotyped by Southern blot using a 5′-flanking probe and *BamH*1-digested genomic DNA [S1]. TSG-p53 mice (C57Bl/6) were purchased from Taconic Farms (Germantown, NY).

Primary fibroblast cultures

Primary fibroblasts were grown from 2 mm of freshly biopsied mouse tails that had been rinsed with PBS. The tails were transferred into growth medium (RPMI1640/10% fetal calf serum/100 units/ml penicillin/streptomycin) and minced using a sterile scalpel blade and the fragments were grown in 4 ml RPMI1640/10% fetal calf serum/100 units/ml penicillin/streptomycin. Fibroblasts growing out of the tail fragments were harvested 6–7 days after biopsy by trypsinization, expanded once for 3 or more days and subjected to analysis.

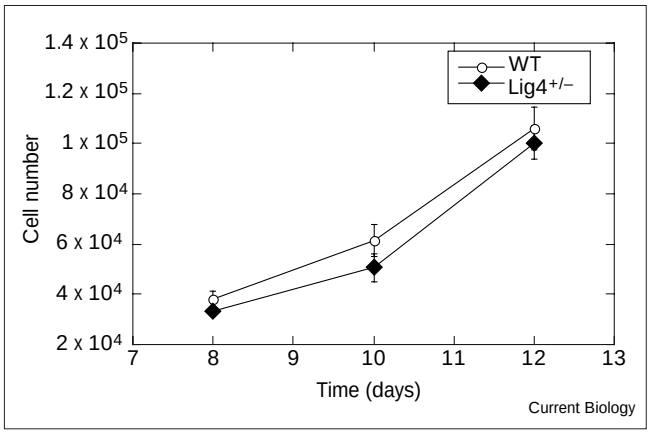
Primary pre-B cell cultures from mice

Long-term proliferating stromal cell and IL-7-dependent pre-B cells were isolated from day 16 fetal liver embryos, as described [S2]. Cell lines were established from individual embryos that were subjected to PCR genotyping as the pre-B cells were growing up. From 12 embryos, pre-B cell lines with all possible genotypic combinations were obtained. Four of 12 embryos carried a total deletion of the Ku86 allele. These four embryos also displayed the dwarfed phenotype. Pre-B cells were grown in IMDM/10% fetal calf serum/0.03% primatone/ 50 μM 2-mercaptoethanol/100 units/ml penicillin/streptomycin/1% conditioned medium obtained from IL-7 secreting cell line J558/IL-7 (kind gift of Dr. Thomas Winkler, University Erlangen, Germany) on a semiconfluent layer of 3000 rad gamma-irradiated ST-2 feeder cells. The pre-B cell lines were analyzed 10–14 days after removal from the mice.

Karyotype analysis

Mouse fibroblasts were seeded onto sterile coverslips and harvested according to standard protocols [S3]. The chromosomes were Wright stained without trypsin treatment [S4]. The metaphase chromosomal abnormalities documented were as follows [S4], and are the same as those described previously [S5]. A chromosome gap is an achromatic region involving both chromatids, where the gaps are less than or equal to the width of a chromatid. A chromatid gap is an achromatic region of one chromatid, where the gap is less than or equal to the width of a chromatid. A chromatid break is where one of the chromatids has a break that is misaligned at a gap that is greater than the width of one chromatid. Regarding Robertsonian translocations, we have counted them as a single chromosomal aberration event. (One could alternatively multiply this number for Robertsonian events by 4, as some have suggested [S6], but which we have not done in Table 1 for simplicity.)

Figure S1



The growth of primary fibroblasts heterozygous for DNA ligase IV is normal with respect to wild-type (WT) fibroblasts. Dermal fibroblasts were harvested from fresh tail biopsies, grown for 7 days, trypsinized, and seeded into T-25 flasks. The number of cells indicated is the average of triplicate cultures. Error bars represent the standard deviation. The number of days designated is the number of days after the initial tail biopsy.

Assessment of Cell Death

The percentage of pre-B cell death in cultures was determined by staining for dead cells with propidium iodide (1 μg of propidium iodide (added from a 100 μg/ml stock) per ml of cell suspension) and calculating the ratio of FL2 positive/negative events. Fibroblasts were counted in triplicate by trypan blue exclusion, and cell death was reported as the percent of dead cells ± standard deviation.

Supplementary references

S1. Frank KM, Sekiguchi JM, Seidl KJ, Swat W, Rathbun GA, Cheng H-L, *et al.*: Late embryonic lethality and impaired V(D)J recombination in mice lacking DNA ligase IV. *Nature* 1998, 396:173-177.

S2. Rolink A, Kudo A, Karasuyama H, Kikuchi Y, Melchers F: Long-term proliferating early pre B cell lines and clones with the potential to develop to surface Ig-positive, mitogen reactive B cells ***in vitro*** and ***in vivo***. *EMBO J* 1991, 10:327-336.

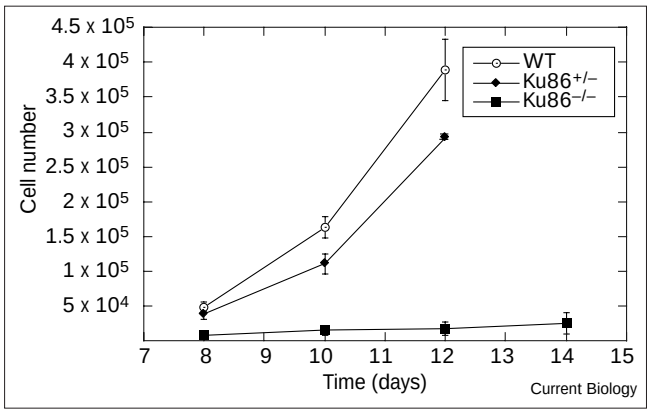
S3. Hsieh C-L: Basic cytogenetic techniques: culturing, slide making, and G-banding. In *Cell Biology: A Laboratory Manual.* Edited by Celis J. San Diego: Academic Press; 1998:391-396.

S4. Mitelman F, ed: *An International System for Human Cytogenetic Nomenclature*. Basel/New York: Karger; 1995.

S5. Martin GM, Smith AC, Ketterer DJ, Ogburn CE, Disteche CM: Increased chromosomal aberrations in first metaphases of cells isolated from the kidneys of aged mice. *Isr J Med Sci* 1985, 21:296-301.

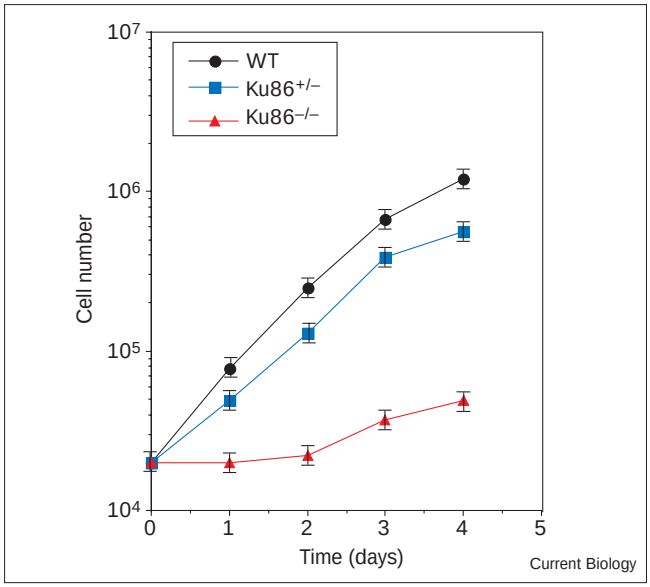
S6. Disney JE, Barth AL, Shultz LD: Defective repair of radiation-induced chromosomal damage in ***scid/scid*** mice. *Cytogenet Cell Genet* 1992, 59:39-44.

Figure S2



The growth of primary fibroblasts from Ku86-null mice, but not heterozygotes, is decreased with respect to wild-type (WT) fibroblasts. Dermal fibroblasts were harvested from fresh tail biopsies, grown for 7 days, trypsinized, and seeded into T-25 flasks. The number of cells indicated is the average of triplicate cultures. Error bars represent the standard deviation. The number of days designated is the number of days after the initial tail biopsy.

Figure S3



The growth of pre-B cells from Ku86 null mice, but not heterozygotes, is decreased with respect to wild-type (WT) fibroblasts. Pre-B cells growing in log-phase were diluted to 2×10^4 cells/ml and plated on a semi-confluent layer of gamma-irradiated ST-2 feeder cells in IL-7-containing growth medium. Cells were harvested from triplicate cultures every other day and counted. The growth is shown as the mean concentration of cells (cell number per ml). The standard deviations were less than 10% of the average cell number from triplicate cultures; this means that the error bars are smaller than the symbol for each of the points and hence the error bars shown are larger than the actual errors.